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(71)(72) Applicants and Inventors: FRYKBERG, Lars [SE/SE]; Stabby Allé 7C, S-752 29 Uppsala (SE). JACOBSSON, Karin [SE/SE]; Stabby Allé 9C, S-752 29 Uppsala (SE).

(74) Agents: BERGANDER, Håkan et al.; Pharmacia AB, Patent Dept., S-751 82 Uppsala (SE).

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(54) Title: PHAGE/PHAGEMID DISPLAY LIBRARY COMPRISING RANDOMLY CLEAVED cDNA/GENOMIC DNA OR CLONED GENE

(57) Abstract

A method for identifying one or more peptides that exhibit(s) biospecific affinity towards a predetermined structure, which method comprises that a) forming a phage/phagemid library by ligating random DNA fragments derived from a cloned gene, cDNA or genomic DNA so as to be in fusion with a gene for a surface protein of a helper phage and b) selecting phage particles binding to the predetermined structure. A phage/phagemid library having individual members that carry a gene for a surface protein fused to DNA fragments that are heterologous to the phage and that are derived from a cloned gene, cDNA or genomic DNA and that the peptides encoded by the fragments are exposed in fusion with the surface protein on the surface of individual phage particle members.

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Phage/phagemid display library comprising randomly cleaved cDNA/genomic DNA or cloned gene.

Technical field

The invention concerns a novel method for identifying peptides that bind to a predetermined target structure. In addition the invention concerns novel virus libraries and a method for producing identified peptides and DNA fragments encoding the peptides. The variation that is found among the individual members of the library of the invention may be derived from DNA-fragments that have been obtained from a cloned gene, cDNA or genomic DNA.

In principle the invention is applicable to virus libraries in general. Since there are important practical advantages associated with bacterial system, previous works within the technical field of the invention have been aimed at bacteriophages for E. coli. In analogy the invention will be illustrated by way of phage/phagemid libraries with E. coli as the host cell. This means that the concept phage/phagemid in connection with the invention shall be interpreted as the corresponding units for virus in general if not otherwise specified. The phagemids employed in the invention carry a gene for a phage/virus surface protein or a modified form thereof capable of being expressed on the surface of the phage/virus.

The expression "peptide" shall be interpreted as oligo- as well as polypeptides with more than 3, preferably more than 5, amino acid residues. The invention is therefore primarily concerned with mapping and selection of peptides having more than 3, preferably more than 5, amino acid residues.

The expression "nucleic acid fragment" and "nucleotide sequence" (DNA-fragment) shall be interpreted as oligo- as well as polynucleotides with more than 5, preferably more than 10 or more than 15, base pairs/bases.

The expression "phage library" shall be interpreted as libraries of phage particles as well as of phagemid particles if not otherwise specified.

Prior art technique and problems associated therewith.

Libraries of short random peptides have for some years been used as stocks from which one has selected peptides of a predetermined binding specificity. In connection with this it

has become popular to express the individual peptide members as fusion proteins with a surface protein of a virus/phage particle. The phage libraries constructed have accordingly carried the variation on the surface of the phage particle (phage display) and on the DNA-level. In the first variants of the technique, the individual oligonucleotide members encoding the respective peptide were fused to native phage DNA. This way of working inherently meant limitations because the oligonucleotides (the insertions) have to be relatively short in order not to destroy the ability of the phage to infect E. coli. Later phage vectors were replaced with phagemid vectors in combination with helper phages, in which system the phagemid vector was the carrier for the intergenic region of the phage and the gene for a phage surface protein. Helper phages, phagemids and surface proteins have primarily been derived from so called filamentous phages with their nose protein (receptor protein) as the preferred surface protein (protein III). Surface proteins that have been utilized so far are normally present in several copies on the same phage particle meaning that when a fused surface protein is exposed on the surface of the phage particle by the aid of a phagemid vector, one or more copies will be present on the same phage particle. For further details within the technical field of the invention see for instance: Smith et al., Science 228 (1985) 1315-17; Cwirla et al., Proc. Natl. Acad. Sci. USA 87 (1990) 6378-82; Devlin et al., Science 249 (1990) 404-406; Stephen et al., J. Mol. Biol. 225 (1992) 577-583; Koivunen et al., J. Biol. Chem. 225 (1993) 577-583; Parmley et al., 73 (1988) 305-308.

An example of a potentially useful surface protein other than Protein III is Protein VIII that is a protein being exposed in several copies on the surface of the same phage as Protein III. Protein VIII has no recognized receptor function associated with the infectivity of the phage (Kay et al., Proc. Natl. Acad. Sci. USA 88 (1991) 4363-66). Another protein that has been employed is Protein VI (Jespers et al., Biotechnology 13 (1995) 378-382)

The phage display technique has also been used for the construction of single chain antibody (ScFv) libraries consisting of phages expressing complete antibody variable

domains (McCafferty et al., Nature 348 (1990) 552-554; and Clackson et al Nature 352 (1991) 624-628) as well as combinatorial antibody fragment (Fab) libraries (Barbas et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7978-7982; Hoogenboom et al., Nucl. Acids Res. 19 (1991) 4133-4137; and Hogrefe et al., Gene 128 (1993) 119-126. The methodology means that certain PCR primers specific for Ig Fab regions are used to make nucleotide sequences coding for the Fab regions. Expression of the sequences in a phage library has enabled researchers to select phages displaying parts of antibodies specifically binding to different antigens. This way of working has the disadvantage that the DNA sequence has to be known in order to perform PCR, which in turn needs access to PCR primers with specificity for Fab-regions in mRNA.

The prior art methods can be summarized as a method employing a phage/phagemid library in order to select peptides binding to a predetermined structure. The methods comprise the steps of:

i. creating a oligo- or polynucleotide library that consists of relatively short synthetic oligonucleotides or of cDNA fragments that have been obtained by PCR amplification of mRNA encoding the Fab regions of immunoglobulins;

ii. creating a library of phage particles by ligating each individual member of the library created in step (i) to the gene encoding a surface protein of the phage particles so that each phage particle will expose on their surface a peptide encoded by the inserted oligonucleotide or by the inserted cDNA fragment, respectively, as a fusion protein with the surface protein;

iii. selecting phages from the library obtained in step (ii) with respect to binding ability to the predetermined structure;

whereafter

iv. the selected phages, if desired, are amplified under selection pressure to contain the fused gene and to express the fusion protein on their surface; and

v. the amino acid sequence(s) of the peptide(s) that is(are) present on the phages selected and that is(are) encoded by the inserted nucleotide sequences (oligonucleotide sequences and cDNA fragment, respectively) and/or the nucleotide sequence encoding said amino acid sequence(s), if desired, is/are determined.

The ligation carried out in step (ii) means that all members of the library created in step (i) are brought into contact with a set of phagemid molecules (phagemid vectors) that have been subjected of restriction enzyme digestion in the same way. The ligation becomes random with respect to which DNA fragment that will be inserted into the phagemid molecule.

The invention

The characteristic feature of the invention is that the DNA fragments which are ligated to the gene encoding the surface protein of the phage are derived from nucleic acid (DNA) corresponding to a cloned gene, cDNA or genomic DNA.

The nucleic acid from which the DNA fragments are derived must contain regions encoding an oligo/polypeptide and can be of eukaryotic or prokaryotic origin. In principle it is not necessary to know anything more about the sequence of the gene or peptide to be selected. cDNA can be prepared by methods known per se from mRNA by the use of reverse transcriptase, e.g. encoding an eukaryotic peptide. By reverse transcribing of eukaryotic mRNA, it is possible to create species and tissue specific phage/phagemid libraries in which the individual members contain different fragments of cDNA. Examples are human specific libraries and libraries specific for selected human tissues, cells and organs, for instance kidney specific libraries.

Random fragments of nucleic acids can be obtained in a number of ways. Preferably one utilizes methods that fragment DNA non-specifically, most preferably completely random or next to random. Examples of appropriate methods are sonication by ultra-sound and digestion with DNase I or other enzymes that non-specifically cleave DNA at different positions independent of the DNA sequence. Potentially it is also possible to utilize

reverse transcriptase and random primers (commercially available) according to standardised methods in order to create random fragments of cDNA from mRNA. The cDNA fragments that are obtained by random primers and reverse transcriptase may, in connection to the invention, be equalized with cDNA fragments formed by random cleavage of cDNA.

The suitable sizes of DNA fragments to be inserted into phage DNA is in the range of 20 - 10 000 base pairs. More narrow ranges are often preferred and may be obtained by selecting the proper methodology for the fragmentation. Broad size ranges may be narrowed by fractionation, for instance by electrophoresis.

The phagemid normally carries a gene encoding antibiotic resistance which enables that the inventive phage/phagemid libraries can be amplified and individual members cloned in a way known per se by culturing the phage/phagemid in the appropriate host cell in the presence of the antibiotic in question. Amplified phagemid vector libraries may be achieved after cultivation and lysis of the host cells used (e.g. E. coli), if these initially have been transformed with phagemids into which the DNA fragments according to above has been ligated. In case phagemid transferred host cells are infected with a helper phage, the cells will form complete phage particles. The result will be a phage library in which some phage particles carry the phagemid vector with the fused gene and other phage particles carry native phage DNA (that lacks the fused gene). On the surface of the phage the native surface protein will be combined with none, one or more molecules fused surface protein. The phagemid vector members in each library will vary with respect to the DNA fragments inserted that for instance may have sizes within the range 20 - 10 000 base pairs. The phage particles of each library will in addition vary with respect to the amino acid sequence (peptide) that is encoded by the inserted DNA fragment. Normally the amino acid sequence will contain between 6 - 3 300 amino acid residues.

Before the DNA fragments are ligated into the phagemid vector, their ends are adapted to the cleavage site that is to be utilized in the vector. In case the fragments have been

formed by sonication or some other method giving various types of ends (various sticky ends and blunt ends), the ends have to be transformed to one and the same type that also match the insertion position of the phagemid. We, ourselves, have selected to make the ends blunt ended before ligation into phagemid vectors that also have been made blunt ended.

At the priority date the preferred phagemid vectors provided the gene for a nose protein (e.g. gene III) as the fusion partner for the inserts (examples 1-3). At the end of the priority year the preferred fusion partner had changed to a gene for a multi copy surface protein like protein VIII (example 4).

The phagemid vectors we have employed carry a DNA sequence called a slippery sequence either before, or after the insertion position of the DNA fragment (and always before the nucleotide sequence coding for the surface protein. The slippery sequence means that the ribosomes may slip and change the reading frame. At the priority date we considered that this could be essential in order to secure a correct reading frame into the inserted DNA and into the nucleotide sequence encoding the surface protein even if the nucleotide sequence of the insertion would give a shift in the same. See examples 1 and 4 under the result for "Analysis of nucleotide sequences". Slippery sequences have been described previously. For a review see Atkins et al., Cell 62 (1990) 413-423. Examples of slippery sequences are sequences providing loops in mRNA, containing so called hungry codons recognized by minor tRNA, especially in tandem (Parker, Microbiol. Rev. 53 (1989) 273-298 and Spanjard et al., Proc. Natl. Acad. Sci. USA, 85 (1988) 7967-7971)), or containing three, four or more consecutively placed identical bases, for instance adenine, or tandem codons.

The phagemid vector we have employed has also had a so called amber stop codon between the insertion site for the fragment and the nucleotide sequence encoding the surface protein. The presence of an amber stop codon may be useful because it will enable that the peptide encoded by the inserted DNA fragment may be directly expressed from the phagemid vector in a host cell lacking amber suppressing ability (the peptide will be obtained in non-fused form). See example 1

"Purification of peptides". The presence of an amber stop sequence in the phagemid vector means that the formation and amplification of our phage particle libraries have to employ host cells that are amber suppressing.

The selection of relevant phages from the library is done i a manner known per se (including future methods) and contemplates that a phage library according to the invention is contacted with the predetermined structure under conditions allowing phage particles exposing a matching amino acid sequence to bind to the structure (normally via biospecific affinity). Thereafter non-binding phage particles are removed from the mixture so that binding phage particles selectively can be released (dissociated) from the predetermined structure. Binding structures can be more or less complex, for instance a single protein, a single epitope, a whole cells, a virus, tissues, a carbohydrate structure, a lipid, an antibody (for instance a polyclonal or monoclonal antibody) etc. We have utilized panning meaning that the phage library is contacted with a surface onto which the binding structure(s) that one desires to select for has (have) been previously affixed. Other alternatives are to present the binding structure in dissolved form and/or native form (for instance cell surface receptors may be presented as cells).

In particular, selection by way of panning often gives a relatively high back-ground (non-specific binding of phages to the predetermined structure or to the surface onto which the structure possibly is affixed). In case a more refined selection is required a rough selection may be completed/ replaced with for instance cloning of the selected phagemids and investigating each clone with respect to content of peptides that have binding ability to a dissolved form of the predetermined structure.

For phage particles that have been selected by the way of their specific binding to the predetermined structure, the sequence for the insert (ligated DNA fragment) and for corresponding peptide may be determined in a manner known per se.

One aspect of the invention is a production method for peptides that bind to a predetermined structure. This aspect

means that one starts with selecting according to the invention a binding peptide, and then utilizes the knowledge of the amino acid sequence of the peptide and/or corresponding nucleotide sequence to produce, in a manner known per se, the peptide as such or a modified form thereof (including fused forms and chemical derivatives and methods to be developed in the future).

A further aspect of the invention is a method to produce a DNA fragment encoding a peptide that binds to a predetermined structure. The method means that one selects phage/phagemids as described above, whereupon the fragment is obtained from the selected phage/phagemid particle in a manner known per se.

A still further aspect of the invention is phage/phagemid libraries having individual members differing in the gene for a surface protein being fused to various DNA fragments that are heterologous to the phage. The DNA fragments contemplated are derived from a cloned gene, cDNA or genomic DNA. The peptides encoded by the inserted DNA fragments are exposed in fusion with the surface protein on the phage particles of the libraries.

The expression "phage/phagemid library" contemplates a library consisting of phage particles and/or phagemid vectors.

At the patent application date, the invention was primarily regarded to be useful for the selection of novel and hitherto unknown binding peptides and for the mapping of binding regions of proteins. A subaspect that is likely to be of extremely great value is mapping of polyclonal antibodies directed against a protein, a virus, a bacterium etc in order to determine immunogenic epitopes, particularly immunodominant epitopes, in more or less complex mixtures of binding structures. See example 3.

The invention is closer defined in the attached patent claims that are part of this specification.

EXPERIMENTAL PART

EXAMPLE 1. FUSION TO GENE III. SCREENING OF THE GENOME OF AN ORGANISM FOR PROTEINS HAVING PREDETERMINED BINDING SPECIFICITIES. MAPPING OF THE FOUND PROTEINS.

INTRODUCTION:

Staphylococcal aureus 8325-4 is known to express protein A, an IgG binding protein, and fibronectin binding proteins, both of which have been earlier cloned (Uhlén et al., J. Biol. Chem. 259 (1984) 1695-1702) and Signäs et al., Proc. Natl. Acad. Sci. USA 86 (1989) 699-703 and Jönsson et al., Eur. J. Biochem. 292 (1991) 1041-48). S. aureus and these two binding abilities were used as a model to test the invention.

MATERIAL AND METHODS

Bacterial strains and helper phage: E. coli strain TG1 (ambersupressing) was used for constructing the library and producing the phage stocks. E. coli strain MC1061 was used for preparing DNA for nucleotide sequencing and for producing recombinant proteins for purification. Phage R408 (Promega) was used as helper phage.

Construction of the library: All DNA manipulations were made with standard conditions. Restriction and modification enzymes were from Amersham (England) and Boehringer-Mannheim (Germany).

Chromosomal DNA was isolated from Staphylococcus aureus 8325-4. The DNA was fragmented by sonication and fragments approximately in size of 100 to 700 bp were isolated by gel electrophoresis. The obtained fragments were made blunt-ended with the T4 DNA polymerase. The phagemid vector pHEN1 (Hoogenboom et al, Nucl. Acids Res. 19 (1991) 4133-37) was digested with Pst1, made blunt-ended and dephosphorylated with calf intestine alkaline phosphatase. Ten µg of the treated vector DNA was ligated with 15 µg chromosomal fragments in a volume of 200 µl. After ethanol precipitation the ligated DNA was dissolved in 20 µl of water and 10 electrotransformations of E. coli TG1, each using 1 µl DNA gave after one hour of non-selective growth in 100 ml Luria Broth (LB) media 9.2×10^6 transformants. The transformants were grown over night in 100 ml LB, 1 % glucose and 50 µg ampicillin/ml. The cells were infected with an MOI of 20 of helper phage R408 in 10 ml LB for one hour and grown for 5 hours in 100 ml LB with 50 µg/ml ampicillin. This gave the resulting library a final titre of 2.6×10^{10} phagemid particles/ml.

Panning procedure: Microtitre plates were coated over night at 4°C with 200 µl of human IgG (500 µg/ml) in 0.05 M $NaHCO_3$, pH

9.7, or bovine fibronectin (100 µg/ml) in the same buffer with the addition of 500 mM NaCl. The wells were then blocked with PBS containing 0.05 % Tween® 20 supplied with 1 % BSA, for 1 hour at room temperature. After washing five times with PBS-Tween, 200 µl of the phagemid library were added to each of three wells and incubated 4 h at 20°C. Following 20 washes with 400 µl PBS-Tween® the bound phages were eluted stepwise with 3 x 200 µl of 50 mM Na-citrate with 150 mM NaCl with a decreasing pH (5.5, 4.5, 3.3, 2.3 and 1.8). The eluates from the three wells were pooled and neutralized by adding 60 µl 2 M Tris pH 8.6. Between 5-50 µl of the eluates were added to 50 µl of fresh E. coli TG1-cells and 100 µl of LB. Following 30 minutes of non-selective incubation at room temperature the suspensions were spread on LA-plates containing 50 µg/ml ampicillin and 2 % glucose and the plates were incubated at 37°C over night.

Repanning: Phages eluted after the primary panning were used to infect TG1 cells. After ampicillin (50 µg/ml) selection on LA-plates the resistant colonies were transferred to 10 ml LB-medium supplied with 2 % glucose and ampicillin (50 µg/ml), and after 4 h growth the cells were collected by centrifugation. The cells were suspended in 1 ml LB-medium and infected with R408 phages (MOI of 20) for 1 h followed by growth for 5 h in 10 ml LB-medium. This procedure routinely gave a phagemid titre between 10^8 - 10^{10} CFU.

Identification of IgG and fibronectin binding clones: Colonies from the ampicillin/glucose-plates were transferred to nitrocellulose filters and incubated on LA-ampicillin plates for 4 h, after which the colonies were lysed in chloroform vapour. The cell debris was washed away in PBS-Tween® and the filters incubated in the same buffer for 30 minutes. The filters were then transferred to a solution containing biotinylated IgG or fibronectin (approximately 5 µg/10 ml) in PBS-Tween and thereafter incubated with horseradish peroxidase-labelled strepavidin (Boehringer-Mannheim, Germany) diluted 1:1000 in PBS-Tween. The enzyme activity was detected using PBS supplied with 4-chloro-1-naphtol and hydrogen peroxide as substrate.

Bovine fibronectin (Sigma, USA) and human IgG were biotinylated with N-hydroxysuccinimide (Sigma) (Bayer et al., 154 (1986) 367-70).

Hybridizations were performed with oligonucleotide probes CB and D in 6 SSC, 0.5 % SDS and 3X Denarth's solution at 37°C. Washing was carried out in 6X SSC and 0.5 % SDS at 60°C. The oligonucleotides were synthesized by Scandinavian Gene Synthesis AB (Swede) and had the sequence: CB: 5'-ACC ACC TGG GTT TGT ATC TTC TTC ATA TTC AAC AAC ATC AGC-3' and D: 5'-GTG TGC TTA TTG AAT CCG TGA ATA TGT GGC ACA CTG TCG-3'.

Determination of the sequence of the displayed inserts: The phagemids were transferred to the E. coli strain MC1061 for isolation of double stranded DNA. The sequences of the inserts were determined from both ends by the dideoxy chain termination method, using the Sequenase version 2.0 DNA sequencing kit from US Biochemical. The oligonucleotides Pe and My used as primers were synthesized by Scandinavian Gene Synthesis AB (Sweden) and had the following sequences: Pe: 5'-TTG CCT ACG GCA GCC GCT GAA-3' and My: 5'-TGC GGC CCC ATT CAG ATC CTC-3'.

The PC-gene program Intelligenetics was used for handling the obtained sequences.

Purification of the peptides: E. coli strain MC1061, which lacks the ambersupressing function and thus produces peptides not fused to protein III, was used for production of IgG binding peptides. Cells containing the phagemid were grown to OD_{550} = 0.5-0.7 in 200 ml LB-medium supplied with ampicillin (50 µg/ml) and glucose (2 %). The cells were pelleted and resuspended in 200 ml LB supplied with ampicillin (50 µg/ml) and 1 mm isopropyl-beta-D-thiogalactoside and grown over night. The cells were pelleted, lysed with lysozyme and the peptides purified by affinity chromatography on IgG Sepharose®. (Pharmacia LKB Biotechnology, Sweden).

Inhibition of IgG binding to S. aureus strain Cowan 1: Staphylococcus aureas Cowan 1 cells were grown over night in 10 ml Tryptic Soya Broth at 37°C. Bacteria were collected by centrifugation and washed three times in PBS-TB (PBS supplied with Tween® 20 (0.05 %) and BSA (0.1 %)) and resuspended in 10 ml PBS-TB.

Purified peptides were mixed with (18 000 cpm) ^{125}I-IgG in a final volume of 100 µl PBS-TB. The mixture was incubated at room temperature for 30 minutes after which 20 µl bacteria were added and the incubation allowed to continue for 10 minutes. The free IgG was removed by washing twice with PBS-TB. The radioactivity in the remaining pellet was measured in a gamma-counter (Searle, USA).

Human IgG was iodinated using IODO-BEADS Iodination Reagent (Pierce, USA) according to the manufacturer's instructions.

RESULTS

Construction of the phage library and panning: DNA from S. aureas 8325-4 was fragmented by sonication and fragments approximately in size of 100 to 700 bp were ligated into the phagemid vector pHEN1. Ligated DNA was introduced into E. coli TG1 by electroporation followed by selection and enrichment of ampicillin resistant bacteria in LB medium. After over night growth the resistant bacteria were centrifuged, resuspended in LB media and infected with R408 helper phage (MOI of 20). The resulting library contained 9.2×10^6 clones and the titre was 2.6×10^{10} phagmid particles/ml. Portions of 0.6 ml from the library were used for affinity panning against the ligands, IgG or fibronectin, immobilized in wells of microtitre plates. The phages were allowed to bind for 2-4 h followed by careful washing with PBS-TB. After stepwise elution with citrate buffer with decreasing pH, eluted phages were allowed to infect E. coli TG1 cells which were spread on ampicillin plates. Ampicillin resistant clones were screened for IgG or fibronectin binding.

After elution at pH 5.5, 4.5 and 3.3 approximately 1-5 % of the clones bound IgG whereas elution at pH 2.3 or 1.8 only few positive clones were released (Table 1).

The frequency of fibronectin binding clones after the primary panning was more difficult to determine since there was an obvious variation in affinity for he labelled fibronectin between different clones. For this reason both biotin labelled fibronectin and DNA probes, oligonucleotides CB and D, covering the two known fibronectin binding domains, were used to detect fibronectin binding clones. Opposite to the situation with the

IgG-binding phages, the frequency of "positive" phage was low at elutions with high pHs, whereas elutions at pH 2.3 and 1.8 resulted in about 10 % fibronectin binding clones (Table 1).

Table 1. The result of four primary pannings with subsequent repanning of the eluted and amplified phages.

pH elution	Percentage binding phages after 1st and 2nd panning							
	First panning				Second panning			
	Exp1 IgG	Exp2 IgG	Exp3 Fib	Exp4* Fib	Exp1 IgG	Exp2 IgG	Exp3* Fib	Exp4* Fib
5.5	1.1	0.4	nd	1.2	14	7.3	nd	8.5
4.5	1.0	1.5	nd	1.1	17.9	13.2	nd	11.0
3.3	6,7	2.2	2.8	0.5	12.2	4.4	12.2	12.9
2.3	nd	0.4	nd	9.2	12.3	5.5	nd	38.6
1.8	nd	0.0	13.0	8.0	1.2	0.0	21.8	20.0

For the secondary panning against IgG, bacteria colonies achieved from infection with phages eluted at pH 5.5, 4.5 and 3.3 were collected and used for preparation of the phage stock. The phage stock used for repanning against fibronectin was collected from elution at pH 3.3, 2.3 and 1.8. The term "binding phages" denotes phages carrying a phagemid which encodes a peptide that binds the ligand.

* The detection of binding clones has been done with two oligonucleotide probes (denoted CB and D) covering parts of the two binding domains encoded by the fnbA gene.

Repanning: Amplified phagemid stocks from the primary IgG and fibronectin pannings were used for a second panning against the ligand. Several different phagemid stocks (from different primary pannings) have been used with similar though not identical results. The results from two repanning experiments against IgG are shown in table 1. In most experiments there was an increase to about 10 % positive clones after the second panning but the result varied between different experiments (ranging about 5 - 20 % positive clones). Repanning against fibronectin gave high frequencies of "positive phages" at low pH, with 20-40 % of the achieved colonies binding fibronectin (table 1).

Analysis of nucleotide sequenc s: Seven IgG binding clones were chosen for further studies and the DNA sequences of the inserts were determined. Six clones, two of which had identical inserts contained parts of the earlier known protein A gene (Löfdahl et al., Proc. Natl. Acad. Sci. USA 80 (1983) 697-701). The inserts differed in size from 169 bp to 648 bp and together they represent the entire sequence encoding the binding region of protein A, which consists of five repetitive domains (Uhlén et al., J. Biol. Chem. 259 (1984) 1695-1702).

The remaining IgG binding clone (Ig4), contained an insert of a so far unknown gene, encoding a second S. aureus IgG-binding protein. This insert encoded peptide of 84 amino acids, the sequence of which was different but with an apparent homology with staphylococcal protein A. Southern blot hybridization, using the insert in clone Ig4 as a probe, showed that the nucleotide sequence of the insert is present in S. aureus strain 8325-4 as well as in other S. aureus strains studied (data not shown).

Sequencing of five fibronectin binding clones showed that all had inserts originating from the staphylococcal gene fnbA which encodes a fibronectin binding protein (Signäs et al., Proc. Natl. Acad. Sci. USA 86 (1989) 699-703). The clones contained sequences covering the two known fibronectin binding domains, the CB domain and the repetitive D domains, respectively (Signäs et al., Proc. Natl. Acad. Sci. USA 86 (1989) 699-703). No clone corresponding to fnbB was found. See table 2.

Table 2. IgG binding and fibronectin binding clones obtained from S. aureus 8325-4.

Protein A			FnBPA		
Clone	Amino acids	Nucleotiodes	Clone	Amino acids	Nucleotides
Ig1	77-222	410-850	KF3	595-682	1898-2164
Ig2	92-164	454-676	KF5	780-864	2452-2707
Ig5	62-277	364-1011	KF9	778-856	2448-2686
Ig6	27-118	260-538	KF10	571-738	1825-2331
Ig8	266-320	977-1145	KF11	580-660	1852-2094

Positions for the amino acids coded by the cloned fragment (to the right in the column for each protein). The numbering starts

with the first amino acid in the signal sequence as determined by (Uhlén et al., J. Biol. Chem. 259 (1984) 1695-1702) (Protein A) and Signäs et al., Proc. Natl. Acad. Sci. USA 86 (1989) 699-703 (FnbA)).

Sequence analysis of the clones showed that all clones had a correct reading frame into the inserts, but surprisingly the reading frame out of the inserts and into the gene for protein III was in no instance correct. Instead, all clones except one analysed so far (in total more than 45 different clones and including also clones from other phagemid libraries) have a +1 or -1 shift in the reading frame out of the insert. This shift depended on the number of nucleotides in the insert. This result was not expected, since the first panning gave a strong selection for phages displaying the binding peptide on the surface, which requires a fusion to the phage protein III. Subsequently, only phages with the insert in the correct reading frame with gene III were expected. This phenomenon suggests two things. Firstly, there must be a strong selection against phages with the insert in correct reading frame with gene III. Secondly, since these phages were affinity purified from a vast majority of other phages, the inserted DNAs must encode a peptide which is displayed at the phage surface. There are two possible explanations to this phenomenon. Either the insert had the correct reading frame initially and mutated in a later stage, or there is a ribosomal slippage resulting in a fusion protein with protein III. For several reasons the first explanations seems very unlikely, for example it is not likely that a mutation that disrupt the reading frame should always occur at the same position, i.e. the 3' end of the insert. Instead ribosomal slippage is the most likely explanation. This has been tested by preparing phage stocks from two of our phagemid clones with the wrong reading frame which then were used in panning experiments. The phage stocks containing our phagemid clones were then highly enriched by the affinity panning. This strongly suggests that the insert in the vector is expressed in fusion with protein III rather frequently. No significant preference for the frequency of +1 and -1 shifts of the reading frame was seen. Between the insert and the start of, or directly after the start of gene III there is a TGA stop

codon present in both the +1 and -1 reading frames, suggesting that the ribosomal slippage occurs before this position. Both +1 and -1 frame shifts are known to occur for example at so called slippery sequences, i.e. a string of four or more single base repeats, for example in bacteriophage T7 (Condron et al., J. Bact. 173 (1991) 6998-7003). For a review see Atkins et al., Cell 62 (1990) 413-23). One such slippery sequence is present in the c-myg tag situated between the insert and gene III and may account for at least part of the ribosomal slippage. Slippage has also been associated with sequences resulting in conformational loops in mRNAs.

Panning with isolated phagemid clones: In order to determine if our phagemid clones with the wrong reading frame produced particles that could be affinity selected, phage stocks from two of the IgG binding clones given in table 2 were collected. These phage stocks were serially diluted and mixed with a constant number of phages from an unrelated phage stock (a phage library made in a similar way but from another bacterial species) followed by panning against IgG and infection of E. coli TG1 cells with the eluted phages. Screening of the achieved colonies for IgG binding, showed a very high enrichment of IgG binding phages (table 3).

Table 3: Panning with a constant amount of phages from an unrelated phage library mixed with phages derived from clones Ig1 or Ig2 in a dilution series.

mixture initially			after IgG panning of mixture	
CFU unrelated	CFU Ig1	CFU Ig2	%Ig1 clones	%Ig2 clones
10^7	10^6	10^6	79.5	81.4
10^7	10^5	10^5	64.0	39.3
10^7	10^4	10^4	31.0	30.0
10^7	10^3	10^3	9.1	15.4
10^7	10^2	10^2	0	2.7*

The CFU value represents the total amount of phagemid particles used in the experiment. These results are average of two experiments. * represents one binding clone out of 18.

Inhibition assays: Peptides encoded by phagemid clones Ig2, Ig4 and Ig6 produced in E. coli MC 1061 were purified on IgG Sepharose® (data not shown). The purified peptides were mixed with ^{125}I-labelled IgG in different concentrations and the binding of labelled IgG to S. aureus Cowan 1 cells was measured. The peptides inhibited binding of IgG to Cowan 1 cells in a concentration dependent manner whereas a crude lysate from 10 ml E. coli MC 1061 cells did not affect the binding (data not shown). Both the binding of the peptide, encoded by clone Ig4 on IgG Sepharose® and the inhibition of IgG binding to S. aureus cells, by the peptide verified that the identified clone encodes a new IgG binding peptide.

EXAMPLE 2. FUSION TO GENE III. MAPPING OF A SINGLE PROTEIN BASED ON A DNA LIBRARY OBTAINED BY FRAGMENTATION OF A CLONED GENE.

INTRODUCTION:

Protein MAG and ZAG are cell surface proteins from the animal streptococcal species S. dysgalactiae and S. zooepidemicus, respectively. The two proteins are closely related to the well known IgG-binding protein G from human group C and G streptococci (Guss et al., EMBO J (1986) 1567-75) and Sjöbring et al., J. Biol. Chem. 266 (1991) 399-405). Besides the binding of IgG and serum albumin which is displayed also by Protein G, the proteins MAG and ZAG also have affinity for the plasma proteinase inhibitor α_2-macroglobulin (α_2M). The cloning and sequencing of the genes for proteins MAG and ZAG have recently described (Jonsson et al., Gene 143 (1994) 85-89) and Jonsson et al., submitted). The α_2M-binding activity of protein MAG and ZAG was localised to the N-terminal part in both proteins. However, overall sequence comparisons showed that there is only limited sequence homology between the two domains.

MATERIALS AND METHODS

Bacterial strains and helper phage: These were the same as in example 1.

Construction of the phagemid library: Plasmid DNA from clones pMAG2 and pSZG40 (Jonsson et al., Gene 143 (1994) 85-89) and Jonsson et al., submitted) encoding the α_2M-binding domain of

protein MAG and the whole protein ZAG, respectively, was purified according to standard procedures (Sambrook et al., (1989) Molecular Cloning: A Laboratory manual, 2nd ed. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press). The purified plasmid DNA was fragmented by sonication and fragments in the range of approximately 50-300 bp were isolated by preparative agarose gel electrophoresis. The isolated fragments were treated with T4 DNA polymerase in order to obtain blunt ends, and then cloned into our modified version, pHENH5, of the phagemid vector pHEN1 (Hoogenboom et al., Nucl. Acids Res. 19 (1991) 4133-37. The modified vector contained an insert made by ligation of two oligonucleotides with the sequences [5'-CCC GGG $(GTG)_6$ CA-3' and 5'-GCA $(CCA)_5$ CCC CGG GTG CA-3'] into the PstI site. Five µg of purified vector DNA was cut with SmaI, treated with calf intestine phosphatase and ligated with 5 µg of gel purified fragments of plasmid DNA from pMAG2 and pSZG40, respectively. After ligation and ethanol precipitation, the ligated material was dissolved in 20 µl dH_2O and 1 µl aliquots were used two electrotransform E. coli TG1 cells. The transformation mixtures were pooled, diluted to 100 ml with LB broth + 2% glucose, and allowed to grow for 1 h without antibiotic selection. A sample was taken for determination of the number of ampicillin resistant clones, and thereafter ampicillin was added to a final concentration of 50 µg/ml and the culture was allowed to grow over night at 37°C. The next day the cells were pelleted, resuspended in 10 ml LB broth and infected with a helper phage R408 at a MOI of 20. After one hour, the cells were diluted to 100 ml with LB broth, ampicillin was added to a final concentration of 50 µg/ml and the culture was incubated for another 5 h. Finally, the bacteria were pelleted and the growth medium containing the phages was sterile filtered and stored at 4°C. The library derived from the zag gene consisted of 1.2×10^5 clones and after propagation the phagemid titer was 9×10^8 CFU. The library from the mag gene consisted of 0.9×10^4 clones and the phagemid titre was 7×10^8 CFU.

Panning of th phage libraries: Microtiter plates (Maxisorp Nunc, Copenhagen, Denmark) were coated over night at 4°C with 200 µl of bovine α_2M (Boehringer Mannheim, Germany) at a

concentration of 100 µg/ml in 0.05 M $NaHCO_3$ pH 9.7. The wells were then saturated with PBS containing 0.05 % Tween® 20 (PBS-Tween®) and 1 % BSA, for one hour at room temperature (RT). After washing with PBS-Tween®, 200 µl of the phagemid library were added to each of three wells and incubated for 4 h at RT. Following the panning, the wells were extensively washed with PBS-Tween® and then stepwise eluted with 200 µl of 50 mM Na-citrate containing 150 mM NaCl of decreasing pH (5.5, 3.3, and 1.8). The eluates from the three wells were pooled and neutralized by the addition of 60 µl 2 M Tris pH 8.6. Fifty µl of the pooled eluates were used to infect 50 µl of E. coli TG1 cells. The total volume was adjusted to 200 µl by the addition of LB broth. After 30 min incubation at RT, the cells were spread on LA plates containing 50 µg/ml ampicillin and 2 % glucose and incubated over night at 37°C.

Identification of α_2M-binding clones: Colonies from E. coli TG1 cells infected with phagemid particles obtained by panning against α_2M, were transferred to nitrocellulose filters which were placed on LA plates containing ampicillin (50 µg/ml). After 4 h the filters were removed and placed for 10 min in chloroform vapour in order to lyse the colonies. The filters were washed in PBS-Tween® in order to remove cell debris and then saturated in the same buffer for 30 min at 37°C. After saturation, the filters were incubated 1 h at 37°C in PBS-Tween® containing horseradish peroxidase (HRP) labelled α_2M at a concentration of 5 µg/ml. The bound α_2M was detected, after thorough washing, by the addition of the HRP substrate 4-chloro-1-naphtol.

Sequencing of the phagemid clones: Clones that were positive in the α_2M-binding were identified on the master plates and grown in small scale for preparation of double stranded plasmid DNA. The DNA obtained was electrotransformed to E. coli MC1061 for preparation of DNA for sequencing. The sequences of the inserts were determined by the dideoxy method, using the Sequenase version 2.0 DNA sequencing kit (US Biochemical) The PC GENE protein and DNA handling program (Intelligenetics) was used for analysis of the sequences.

Inhibition of α_2M-binding to S. zooepidemicus c lls: Lyophilized S. zooepidemicus cells of strain Z5, from which the

zag gene was cloned, were coated into microtiter wells (Rantamäki et al., J. Dairy Res. 59 (1992) 273-85). After saturation of the wells, 200 µl HRP-conjugated α_2M (10 µg/ml) in PBS-Tween® + 2 % BSA was added to the wells together with increasing amounts of lysates made from E. coli MC1061 cells expressing peptides representing each of the four binding domains. A control lysate from a clone containing only the vector was included in the assay. After incubation for 1 h at RT, the wells were washed repeatedly with PBS-Tween® and 100 µ l 3,3',5,5'-tetramethylbenzidine (Boehringer Mannheim, Germany) was added as substrate for the enzyme. The reaction was stopped after 5 min by the addition of 100 µl of 1 M HCl and the plates were read in ELISA reader at 450 nm.

Results:

Nucleotide sequences of the inserts: Out of the seven sequenced clones derived from the mag gene library, six were unique. The corresponding figures for the clones derived from the zag gene were 11 unique clones. All inserts were derived from the domains of the respective genes, that wee earlier defined as encoding the α_2M-binding activity (Jonsson et al., Gene 143 (1994) 85-89) and Jonsson et al., submitted). The localization of the different clones is given in table 1. The clones derived from the library of the zag gene cover either of the two repeats, R1 and R2, which divides the originally identified binding area into two sites. However, there is an overlap between clones covering the R1 and R2 repeats, respectively, which in the shortest case is 18 nucleotides long. The deduced amino acid sequences from the colonies derived from the zag gene differ in length from 91 to 66 amino acids.

Five of the six clones derived from the mag gene cover the first α_2M-binding site of the domain, which is slightly homologous to the repeats coded by the zag gene. One of the clones from the mag gene was located further downstream, which also divides the α_2M-binding domain of protein MAG into two separate α_2M-binding sites. See table 4.

Table 4. α_2M-binding binding clones obtained from the ZAG gene.

Positions for the amino acids coded by the cloned fragment (left column in the columns for each protein). The numbering starts with the first amino acid in the signal sequence.

ZAG		MAG	
Clone	Amino acids	Clone	Amino acids
Z1	84-174	M1	29-111
Z2	26-110	M2	41-113
Z3	89-174	M3	21-114
Z4	101-169	M4	46-131
Z5	92-164	M5	15-112
Z12	25-110	M7	126-192
Z14	99-176		
Z15	89-169		
Z16	36-106		
Z17	98-169		
Z18	99-169		

Inhibition of α_2M-binding to S. zooepidemicus: Four clones representing the different α_2M-binding sites as defined by nucleotide sequencing, were chosen for inhibition studies (two represented the binding sites from protein MAG and two represented the two different sites from protein ZAG). A clone, containing the vector without any insert, was included as a control. All clones encoding α_2M-binding motifs efficiently inhibited the binding of α_2M to S. zooepidemicus strain Z5 cells immobilized in microtiter wells. The control lysate also slightly inhibited the binding to α_2M at higher concentrations.

The lysates were run on SDS-PAGE and tested in a Western blot before they were used in the inhibition assays. The protein contents of the lysates were in the same range and only clones expressing parts of the mag and zag gene were detected in Western blots using HRP-labelled α_2M (data not shown).

EXAMPLE 3. FUSION TO GENE III. MAPPING OF POLYCLONAL ANTIBODIES DIRECTED AGAINST HUMAN FIBRONECTIN. SCREENING OF DNA-FRAGMENTS DERIVED FROM cDNA.

Preliminary experiments and results: The starting DNA was a plasmid clone containing cDNA which encodes the human fibronectin protein (Dufour et al., Exp. Cell. Res. 113 (1991)

331-38). This cDNA was used to made a phage display library according to the invention.

The library was panned against affinity purified antibodies directed against human fibronectin, developed either in goat (Ninolab) or in rabbit (Sigma). The phages were eluted with decreasing pH and used to infect E. coli TG1 cells. Bacterial colonies expressing antibody binding epitopes were isolated and the inserts of the phagemids were sequenced. The inserts of the phagemid clones obtained by panning against goat antibodies were clustred in one major site in fibronectin (six clones out of 10), from approximately nucleotides 2900 to 3200 in the cDNA. Two other epitopes were also found, one located approximately from nucleotides 2600 to 2800 in the cDNA (three clones out of 10) and the other from nucleotides 1820 to 2100 (one clone). The rabbit antibodies also recognized one major epitope on the fibronectin protein since 22 clones out of 24 had an insert of cDNA from approximately nucleotides 2960 to 3140. Two other epitopes were also found for rabbit antibodies and were identified as inserts of approximately nucleotides 2500 to 2700 and 2770 to 2900 in the cDNA.

This study has recently started and the results are not yet complete.

EXAMPLE 4. FUSION TO GENE VIII. SCREENING OF THE GENOME OF AN ORGANISM FOR PROETINS HAVING PREDETERMINED BINDING SPECIFICITIES

MATERIALS AND METHODS

Bacterial strains and helper phage: These were the same as in example 1.

Construction of the phagemid vector: All DNA manipulations were performed using standard methods, with an exception for ligations, for which Ready to Go T4 DNA ligase (Pharmacia Biotech AB, Sweden) was used according to the manufacturer's instructions. Restriction enzymes was from Amersham and Boehringer-Mannheim. The oligonucleotides were synthesized by Scandinavian Gene Synthesis AB (Falkenberg, Sweden).
The vector was constructed from pHEN1 (Hoogenboom, Nucl. Acids Res. 19 (1991) 4133-4137) with a modification in two steps. First, a tail encoding 6 histidine residues was introduced to enable purification of the polypeptides. The oligonucleotides,

5`-GCA CCA CCA CCA CCA CCA CCC CGG CTG CA-3´and 5`-CCC GGG GTG GTG GTG GTG GTG GTG CTG CA-3´, encoding the histidine tail and a SmaI site, where inserted into the PstI restriction site in the pHEN1 vector. In addition, this linker introduced a frame shift mutation in order to decrease the expression of gene III. This modified pHEN1 vector was called pHENH6. Second, gene III was exchanged for gene VIII to form pG8H6. Gene VIII was obtained from phage R408 by PCR amplification using the primers 5´-GGC AAT AAG CTT GAG GGT GAC GAT CCC GCA AAA-3´and 5`-TTT CCC GAA TTC ATC GGT TTA TCA GCT TGC TTT CG-3´. The fragment was purified by preparative gel electrophoresis and digested with EcoR1 and HindIII. Similarly a fragment of pHENH6 containing the polylinker, the histidine tail and c-myc tag was amplified using the primers 5´-TTT CCC AAG CTT CTA TGC GGC CCC ATT CAG ATCC-3´ and 5´-TTG CCT ACG GCA GCC GCT GAA-3´and subsequently gel purified and digested with NcoI and HindIII. The two fragments were ligated into the pHENH6 vector, previously digested with EcoRI and NcoI and dephosphorylated with calf intestine alkaline phosphatase. After transformation into E. coli MC1061, plasmid DNA was prepared and the sequence was confirmed by restriction analysis as well as DNA-sequencing through both inserts using pUC/M13 forward and reverse primers.

Construction of the library: The library was constructed essentially as described in example 1. In short chromosomal DNA from staphylococcus aureus strain 8325-4 was randomly fragmented by sonication and fragments, approximately 300-800 bp in size, were isolated by preparative gel electrophoresis. The fragments were made blunt ended with T4 DNA polymerase and ligated into the pG8H6 vector, previously digested with SmaI and dephosphorylated with calf intestine alkaline phosphatase. The ligation was made with Ready to Go ligation kit (Pharmacia Biotech AB, Sweden) with 5 µg of fragments divided in 5 tubes. The ligated material was phenol- and chloroform-extracted, EtOH precipitated twice, and dissolved in 10 µl of H_2O. Ten electrotransformations into E coli TG1 (1 µl in each) gave $1x10^7$ ampicillin resistant transformants. After growth over night at 37°C an aliquot of the transformants (2 ml) were infected with helper phage R408 at MOI 50 and incubated for 15 min at 37°C. Thereafter the infected cells were mixed with 100

ml 0.5 % soft agar and poured on 20 LA-plates with ampicillin (50 µg/ml). After incubation over night the phage particles were eluted by vigorous shaking of the soft agar in 100 ml LB for 3 hours. The suspension was cetrifuged at 40 000g for 10 min, the supernatant sterile filtered and the stock was frozen in aliquots, which were used for the pannings. After centrifugation and sterile filtration, the titre of the library was determined by infection of E coli TG1 cells followed by plating on LA-plates with ampicillin.

Panning procedure: Microtiter wells (Maxisorp, Nunc) were coated with either human IgG (100 µg/ml, Pharmacia AB, Sweden), human serum albumin (100 µg/ml, Sigma, St Louis, USA), fibronectin (100 µg/ml, Sigma or Bional Ltd, Estonia) or Fibrinogen (100 µg/ml, Sigma) and blocked with 1 % BSA and 0.05 % Tween® in PBS. Two hundred µl of the library was added to each of three wells and incubated for 4 hs at room temperature (RT). Thereafter the wells were washed extensively with PBS containing 0.05 % Tween®, twice with 50 mM Na-citrate/140 mM NaCl pH 5.5 and finally the bound phage were eluted stepwise in the same buffer with decreasing pH (3.7 and 2.1). The eluates from the three wells were pooled and neutralized with 2 M Tris pH 8.7. Aliquots of the eluates were used to infect E coli TG1 cells which then were grown overnight on LA plates (LB-medium with 1.5 % agar) supplied with 2 % glucose and 50 µg/ml ampicillin.

The colonies obtained after infection of TG1 cells with the eluted phage at pH 3.7 and 2.1 in the primary panning, were collected and infected with helper phage R408 (10^{10} pfu) for the production of enriched stocks. Thereafter the infected bacteria were mixed with 5 ml 0.5 % soft agar and poured on the LA-plate with ampicillin. After incubation over night the phage particles were eluted by vigorous shaking of the soft agar in 5 ml for 3 hours. The suspension was centrifuged at 40,000g for 10 min, the supernatant was sterile filtered and used as a stock for panning as described above. This procedure routinely gave a phagemid titre of 10^{9}-10^{10} colony forming units (cfu) per ml.

Nucleotide sequencing of the displayed inserts: Plasmid DNA was prepared using Wizards™ Minipreps (Promega) and the sequences was determined as described in example 1.

Identification of IgG binding: This was made essentially as described in example 1 except for the detection being carried out with IgG labelled with horseradish peroxidase (HRP).

RESULTS AND DISCUSSION

Construction of the gene VIII vector and the phage library: The data presented in example 1 indicated that ribosomal slippage is required to get expression of functional pIII-fusion peptides. The placing of the His-tag in front of the expected fusion peptide is a safety measure to guarantee fusion peptides containing the His-tag.

A library was constructed from chromosomal DNA from S aureus 8325-4 with fragments ranging in size approximately 300 to 800 bp using the pG8H6 vector. The resulting library consisted of 10^7 clones and had, after infection with helper phage, a titre of $2x10^{10}$ cfu.

Panning of the library against different ligands: The pannings were repeated several times and the results from one typical experiment are shown in table 5. A fraction of the phage eluted at pH 2.1 and 3.7, corresponding to 100-1000 cfu, was used for infections of E coli TG1 cells to obtain phage stocks specific for a certain ligand, which then were used for the second panning. To confirm that any enrichment in the panning was due to affinity selection for the ligand, and not depending on an unspecific interaction of the phages to the plastics, each stock was repanned against the specific ligand and against two independent ligands. The results in Table 5 show that it is possible to achieve a tremendous enrichment when the repanning is made against the same ligand as the primary panning (IgG and fibrinogen, 10^4-10^5 fold increase in the number of eluted phagemid particles). This is a specific enrichment since the same phage stock panned against an unrelated ligand gave more than a 1000 fold lower number of bound phagemid particles. However, the different pannings and subsequent repannings against fibronectin gave a great increase in number of bound phagemid particles. After a repanning against fibronectin only

a small increase, about five fold, in number of bound phagemid particles was obtained. However, the same phage stock panned against an unrelated ligand gave a significantly lower number of bound phagemid particles, suggesting that the binding is specific. After the primary panning against HSA only few phagemid particles were eluted and repanning did not significantly increase the number, which suggests that no enrichment occurred when the panning was made against ligands for which the bacteria do not encode a specific receptor.

After the primary and secondary pannings against IgG the ampicillin resistant bacterial clones, obtained by infection with the eluted phages, were screened for binding to peroxidase labelled IgG. The result was difficult to interpret, and was clearly different from the similar screening described in example 1. Only a few of the colonies obtained after the primary panning gave a clear and strong signal when assayed for binding of labelled IgG. However, several colonies seemed to have a weak binding capacity not easily distinguishable from the background. In screenings of colonies obtained after the secondary panning, almost 100 % of the clones gave a weak IgG binding signal (data not shown), opposite to the results presented in example 1.

Table 5.

Ligand 1st panning	Ligand 2nd panning	Cfu/ml in the eluate 1st panning pH 3.7	1st panning pH 2.1	2nd panning pH 3.7	2nd panning pH 2.1	Correct/ sequenced[1]
IgG	IgG	2.2×10^3	8.6×10^2	3.2×10^7	4.5×10^7	16/16
IgG	fbn[2]			1.2×10^4	1.4×10^4	
IgG	fbg[3]			8.4×10^3	1.0×10^3	
fbg[3]	fbg[3]	1.3×10^3	4.8×10^2	2.2×10^7	1.9×10^7	13/16
fbg[3]	fbn[2]			2.3×10^3	1.7×10^4	
fbg[3]	IgG			4.0×10^4	1.0×10^4	
fbn[2]	fbn[2]	3.1×10^4	1.3×10^4	1.4×10^4	6.6×10^4	12/16
fbn[2]	fbg[3]			2.9×10^3	6.4×10^3	
fbn[2]	IgG			1.7×10^3	7.8×10^2	

HSA	HSA	3.2×10^2	40	8.0×10^2	1.8×10^2

1) Number of inserts derived from a gene known to encode the binding activity in question/number of clones picked at random.

2) Fibronectin 3) fibrinogen

Nucleotide sequence analysis of affinity selected clones: 16 colonies were picked at random after the secondary panning against each of IgG, fibrinogen and fibronectin and sequence analysed. See table 5. For the IgG binding clones 16 out of 16 inserts were derived from the gene encoding Staphylococcal protein A. For the fibrinogen binding clones 13 out of 16 contained inserts encoding known fibrinogen-binding proteins, with one clone representing the fib gene and twelve the coa gene (Bodén et al., Mol. Microbiol. 12 (1994) 599-606 and McDevitt et al., Mol. Microbiol. 11 (1994) 237-248). For fibronectin the corresponding number was 12 out of 16 with the fnbA gene being represented by 11 clones and the fnbB gene by one clone.

Interestingly, of the 41 clones with correct inserts, only one exhibits the shift in reading frame between the insert and the vector as described in example 1. All the other clones have the right reading frame in conjunction between the insert and the c-myc tag. However, the reading frame from the PelB leader entering the insert is in all these cases out of frame, i.e. there is a shift in reading frame to either +1 or -1 between the PelB sequence in the vector and the insert. This result was not expected, and as in example 1 we suggest that there is a strong selection against clones with the right reading frame of the insert in conjunction with the vector sequences. Since there is a strong selection for clones expressing polypeptides with binding affinity for the different ligands used in the pannings, the insert must be expressed in fusion with the major coat protein and thereby located to the phage surface. Probably, ribosomal slippage is the explanation to this phenomenon (cf. example 1) and may be caused by the 6 identical codons in tandem inserted as the His tag into the starting vector. In addition the construction of the vector created four cytosines in a row in the SmaI-site. We cannot explain why an exchange of gene III for gene VIII results in selection for

clones with the wrong reading frame into the insert instead of out of the insert. However, this result agrees well with our observation that after the first panning against IgG, clones expressing either strong or weak binding activity were found, but after the second panning almost exclusively clones with only weak binding activity were observed. This weak binding capacity was not due to expression of polypeptides with low affinity since they all contained several IgG-binding repeats of protein A, but rather to a low expression level since ribosomal slippage is a requirement for expression of the polypeptide encoded by the insert. In addition, we have sequenced five clones which after the first panning gave a strong signal for binding of HRP-labelled IgG, and in those cases the inserts either had the right reading frame in conjunction with the PelB leader or had their own endogenous promotor from which the expression was controlled.

PATENT CLAIMS

1. A method for identifying one or more peptides that exhibit biospecific affinity towards a predetermined structure, said method comprising
   i. forming a phage/phagemid library by ligating DNA fragments derived from a cloned gene, cDNA or genomic DNA in fusion with a gene for the surface protein of a helper phage, and
   ii. selecting phage particles that bind to the predetermined structure.

2. The method according to claim 1, **characterized** in that the fragments have been produced by random cleavage of DNA, for instance by sonication.

3. The method according to anyone of claims 1-2, **characterized** in that the cDNA from which the DNA fragments are derived has been obtained by reverse transcription of mRNA of a uni- or multicellular organism, e.g. a mammal such as a human and then the mRNA in particular can be derived from a certain tissue.

4. The method according to anyone of the preceding claims, **characterized** in that the DNA fragments have sizes within the range of 20 - 10 000 base pairs.

5. The method according to anyone of the preceding claims, **characterized** in that the phages/phagemids, into which the DNA fragments have been ligated, have a slippery sequence which:
   a. is located either before or after the insertion site for the DNA fragments, and
   b. secures a correct reading frame for the insert and the nucleotide sequence encoding the surface protein even if the insertion of the DNA fragment gives a shift in the reading frame.

6. The method according to claim 5, **characterized** in that the slippery sequence has three, four or more repeated and

identical bases immediately following after one another, and/or hungry codons and/or tandem hungry codons and/or tandem codons.

7. The method according to anyone of the preceding claims, **characterized** in that the phages/phagemids have an amber stop sequence between the position for the inserted DNA fragment and the sequence encoding the surface protein.

8. The method according to anyone of the preceding claims, **characterized** in that the fused gene is located on a phagemid or a modified form thereof, which phagemid is utilized in combination with a helper phage that carries the gene encoding a native form of the surface protein in order to produce phage particles in E. coli, with the provision that in case the phage/phagemid contains an amber stop sequence the host cell is an E. coli strain that is amber suppressing.

9. A method for producing a peptide that binds to a predetermined structure by the way of biospecific affinity, **characterized** in

i. **identifying** according to anyone of claims 1-8 a peptide that binds to the structure;

whereafter

ii. the identified peptide or a modified form thereof that has binding ability to the predetermined structure is produced.

10. The method for producing a polypeptide according to claim 9, **characterized** in that the nucleotide sequence or a degenerative variant thereof encoding the selected peptide is expressed in the appropriate host cell.

11. A phage/phagemid library having individual members that carry a gene for a surface protein fused to DNA fragments that are heterologous to the phage and that are derived from a cloned gene, cDNA or genomic DNA, and that the peptides encoded by the fragments are

exposed in fusion with a surface protein on the surface of individual phage particle members.

12. The library according to claim 11, **charact rized** in that it is a phagemid library.

13. The library according to claim 12, **characterized** in that it is a library of phage particles.

14. The library according to anyone of claims 11-13, **characterized** in that the DNA fragments have been obtained by cleavage, for instance by sonication, of a cloned gene, cDNA or genomic DNA, said cDNA, for instance, having been obtained by reverse transcription of mRNA from a uni- or multicellular organism, such as a mammal including human and then from a specific tissue.

15. The library according to anyone of claims 11-14, **characterized** in that the sizes of the DNA fragments are selected within the range of 20-10 000 base pairs.

16. The library according to anyone of claims 11-15, **characterized** in that the fused gene contains a slippery sequence that
    a. is positioned either before or after the insertion position for the DNA fragment, and
    b. secures a correct reading frame for the insert and the nucleotide sequence encoding the surface protein even in case the insertion of the DNA fragment results in a shift in the reading frame.

17. The library according claim 15, **characterized** in that the slippery sequence has three, four or more consecutively repeated identical bases, or several identical codons in a row or hungry codons.

18. The library according to anyone of claims 11-15, **characterized** in that the fused gene has an amber stop

sequence between the inserted nucleotide sequence and the sequence that encodes the surface protein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00515

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/53, G01N 33/68, C12N 7/01, C12N 15/63
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, SCISEARCH, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 154, MEDLINE, Dialog accession no. 07541286, Medline accession no. 91060286, Greene WK et al: "Antigenic analysis of group I house dust mite allergens using random fragments of Der p I expressed by recombinant DNA libraries", & Int Arch Allergy Appl Immunol (SWITZERLAND) 1990, 92 (1) p 30-8 --	1-4,7-15,18
X	Dialog Information Services, file 154, MEDLINE, Dialog accession no. 07063005, Medline accession no. 89365005, Muller N et al: "Application of a recombinant Echinococcus multilocularis antigen in an enzyme-linked immunosorbent assay for immunodiagnosis of human alveolar echinococcosis", Mol Biochem Parasitol Sep 1989, 36 (2) p 151-9 --	1-4,7-15,18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1995	08 -09- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00515

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 9006132 A1 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE), 14 June 1990 (14.06.90), page 4, line 10 - line 17, claims --	1-4,7-15,18
A	J. MOL. BIOL., Volume 222, 1991, F. Felici et al, "Selection of Antibody Ligands from a Large Multivalent Exposition Vector" page 301 - page 310 --	1-4,7-15,18
A	PROC. NATL. ACAD. SCI., Volume 91, January 1994, R. P. Kandapal et al., "Construction of libraries enriched for sequence repeats and jumping clones, and hybridization selection for region-specific markers" page 88 - page 92 --	1-18
A	US 4508823 A (R.H. OLSEN), 2 April 1985 (02.04.85) --	1-18
A	IMMUNOLOGICAL REVIEWS, Volume 130, 1992, E. Söderlind et al, "Phage Display Technology in Antibody Engineering: Design of Phagemid Vectors and in vitro Maturation Systems" page 109 - page 124 --	1-18
A	National Library of Medicine (NLM), database Medline, NLM accession no. 92345531, Fu YX et al: "On the design of genome mapping experiments using short synthetic oligonucleotides", Biometrics 1992 Jun; 48 (2): 337-59 --	1-4,7-15,18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00515

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	PROC.NATL.ACAD.SCI., Volume 87, August 1990, Steven E. Cwirla et al, "Peptides on phage: A vast library of peptides for identifying ligands" page 6378 - page 6382 --	1-4,7-15,18
A	SCIENCE, Volume 249, 1990, James J. Devlin et al, "Random Peptide Libraries: A Source of Specific Protein Binding Molecules" page 404 - page 406 --	1-4,7-15,18
P,X	National Library of Medicine (NLM), database Medline, NLM accession no. 95130942, Wang LF et al: "Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5", J Immunol Methods 1995 Jan 13; 178 (1): 1-12 -- --------	1-4,7-15,18

INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/95

International application No.
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		FR-A- 2640877	29/06/90
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		US-A- 4508824	02/04/85
		US-A- 4508827	02/04/85
		EP-A,A,A 0081237	15/06/83